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Inhibition of Prostate Cancer Skeletal Metastases by Targeting Cathepsin K

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14. ABSTRACT

We have previously shown that CatK is expressed not only by osteoclasts but also by prostate cancer (PCa) cells and stromal cells. Zoledronic acid (ZA), a bisphosphonate which exerts beneficial effects in PCa patients with bone metastases, reduces both pain and skeletal related events. We hypothesized that combination of a bisphosphonate with the CatK inhibitor, through different inhibitory mechanisms, could diminish PCa progression in vivo. Accordingly, PCa C4-2B cells were inoculated into the tibiae of SCID mice. The mice were randomized into the following treatment groups (n=10/group): ZA, CatK inhibitor, the combination of ZA and CatK inhibitor, and saline vehicle alone. Test drugs were initiated at 4 weeks after the tumor cell inoculation and treatments were continued for 8 weeks. We found that CatK inhibitor significantly reduced skeletal tumor burden as determined by both tumor volume vs soft tissue volume ratio and serum PSA levels. In contrast, ZA less effectively diminished the skeletal tumor volume. ZA failed to decrease PSA levels. Importantly, combinations of the two agents were more effective in decreasing tumor volume than any single agent. Therefore, we show for the first time that a CatK inhibitor diminishes PCa growth in bone and the inhibitory effects are enhanced in combination with ZA

15. SUBJECT TERMS
Cathepsin K inhibitor, Zoledronic Acid, prostate cancer, bone metastasis, animal model

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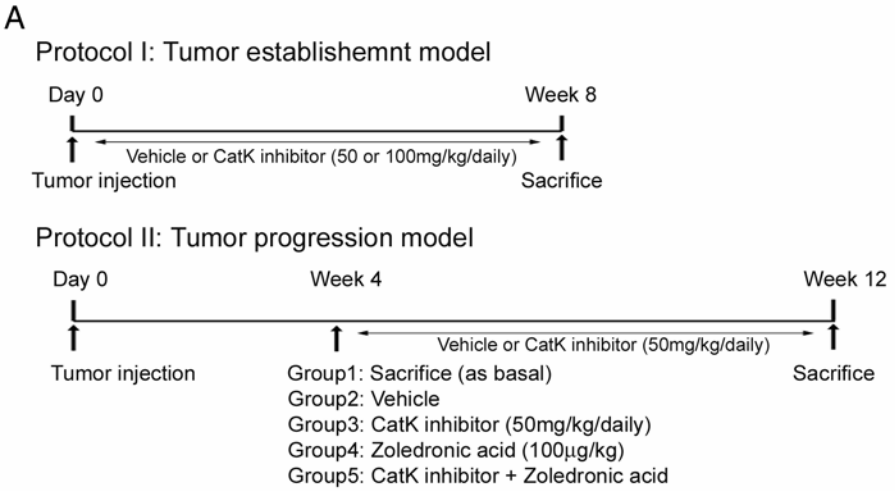
Introduction

The **subject of this study** is: "Inhibition of prostate cancer (PCa) skeletal metastases by targeting cathepsin K". PCa is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States. New treatments are urgently needed for patients known to have bone metastases and those who are at high risk for having bone metastases in PCa patients. Cathepsin K (CatK) is a cystine protease enzyme that can degrade collagen I, the organic matrix of the bone. CatK was found in breast cancer cells that are PCaable of bone resorption and CatK message RNA was also detected in PCa cell lines and in primary PCa and metastases. Importantly, the CatK expression in bone metastases was significantly higher than in primary PCa, while CatK in normal prostate tissues were negative. These findings suggest that CatK may play a critical role in tumor bone metastasis. **The purpose of this project is to identify a clinically relevant strategy to inhibit prostate cancer skeletal metastasis.** The **specific scopes** of this research are: 1) To determine the effects of CatK inhibitor and Zometa (ZA) alone or in combination on the establishment of PCa growth in bone, and determine if these effects are synergistic. 2) To determine the effects of CatK inhibitor and ZA alone or in combination on the progression of PCa growth in bone, and determine if these effects are synergistic.

Body

This grant was transferred from University of Pittsburgh to University of Michigan in April 2010. Although it took about a year for this transfer process, we have finished all major tasks that involve a lot of animal work. Our objectives in this grant are to determine the effects of CatK inhibitor and zoledronic acid (ZA) alone or in combination on the establishment (Task 1) and progression (Task 2) of PCa growth in bone, and determine if these effects are synergistic. In the first year of study, we confirmed the expression of CatK in PCa cell lines using immunohistochemical staining, RT-PCR, and Western blot, and ELISA on cultured media (CM) to measure CatK in cell supernatants. We also collected CM from C4-2B cell culture to study CatK inhibition of bone resorption in vitro. Results showed C4-2B-induced bone resorption was significantly diminished by CatK inhibitor. We then investigated the inhibitory effect of CatK on PCa cell invasion using C4-2B cells cultured in transwell chambers. Results showed invasion was significantly reduced in a dose-dependant manner in the CatK inhibitor treatment groups. Finally, C4-2B cells were injected into the tibiae of SCID mice, and then the animals received either vehicle, low or high doses of CatK inhibitor for eight weeks. Treatment with vehicle or inhibitor was provided either at the time of tumor cell injection or 4 weeks after, representing a tumor establishment (Figure 1A, protocol I) and progression model (Figure 1A, protocol II), respectively. Mice were sacrificed at 12 weeks, and X-ray, histological staining were performed and PSA level determined. In the tumor establishment model, CatK inhibitor significantly prevented the establishment of mixed osteolytic/osteoblastic tibial tumors such as were observed in vehicle-treated animals. In the progression model, CatK inhibitor diminished tumor-induced bone lesions.



Based on the findings in the 1st year, in the 2nd year, we included treatment of experimental animals with ZA alone or in combination with CatK inhibitor (Figure 1A, protocol II). Here, we report: ***CatK Inhibitor Prevents Establishment and Retards Progression of PCa Tumor in Murine Bone***

Briefly, to determine if inhibition of osteoclastogenic activity by the CatK inhibitor could prevent establishment or retard progression of PCa *in vivo*, we directly injected C4-2B cells into the tibia of SCID mice. The experimental protocols are summarized in figure 1*A*. In protocol 1, the mice were administered orally either CatK inhibitor or vehicle either at the time of tumor cell injection. In protocol 2, four weeks after the tumor cells injection, the mice were randomized into groups: ZA, CatK inhibitor, the combination of ZA and CatK inhibitor, and saline vehicle alone. Test drugs were given for 8 weeks. We found that CatK inhibitor significantly inhibited the establishment and development of PCa tumor in bone (Figure 1*B* and *C*). C4-2B caused dominantly osteolytic areas with some areas of osteoblastic activity (Figure 1*B*). It significantly reduced tumor volume (Figure 1*C*), serum PSA levels (Figure 1*D*) and osteoclast formation at bone-tumor interface (Figure 4*E*) compared to the vehicle-treated animals. These results indicated tumor burden was reduced by administration of the CatK inhibitor. In contrast, ZA less effectively diminished the skeletal tumor volume compared to the administration of the CatK inhibitor (Figure 1*C*). Interestingly, ZA failed to decrease PSA levels in these animals (Figure 1*D*). The inhibitory effects of the CatK inhibitor were enhanced in combination with zoledronic acid treatment (Figure 1*B* to 1*D*).

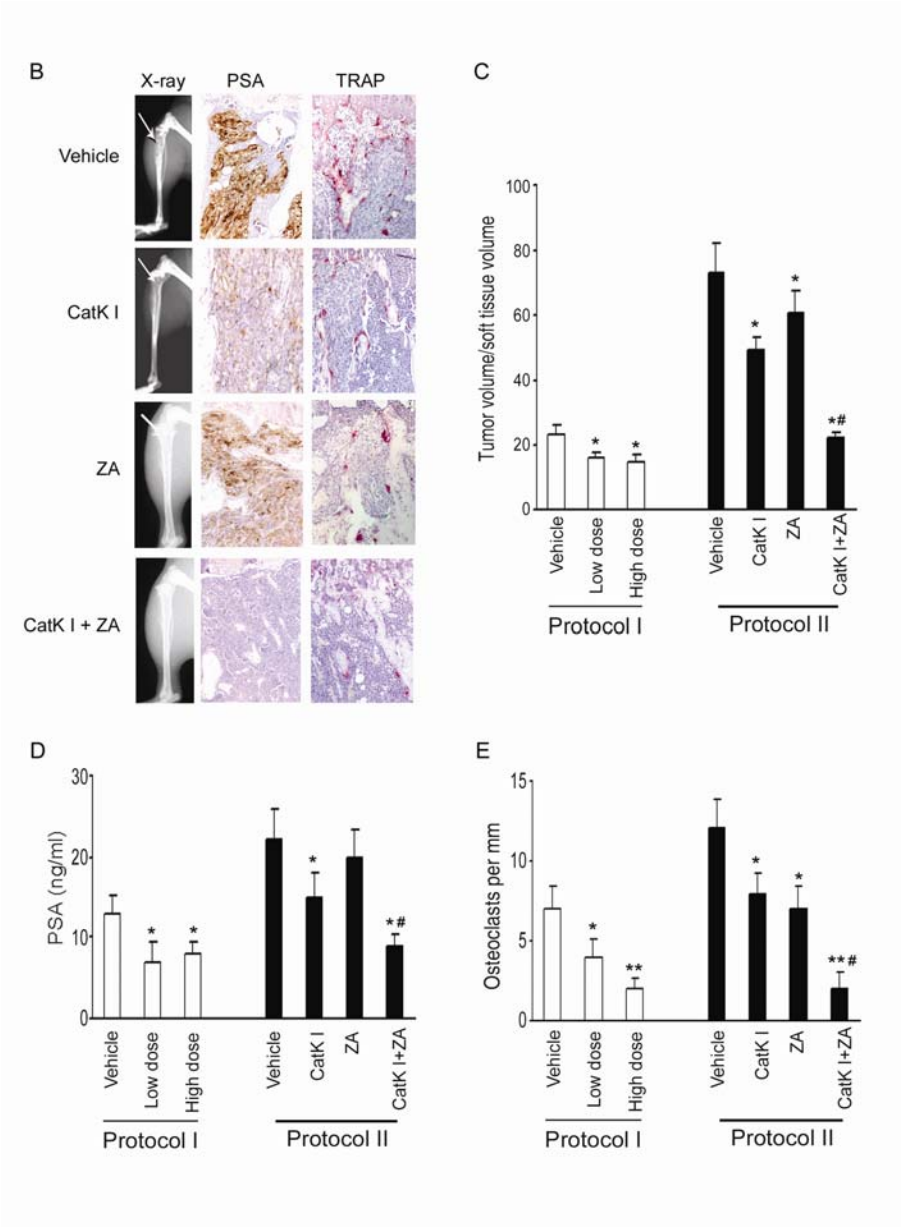
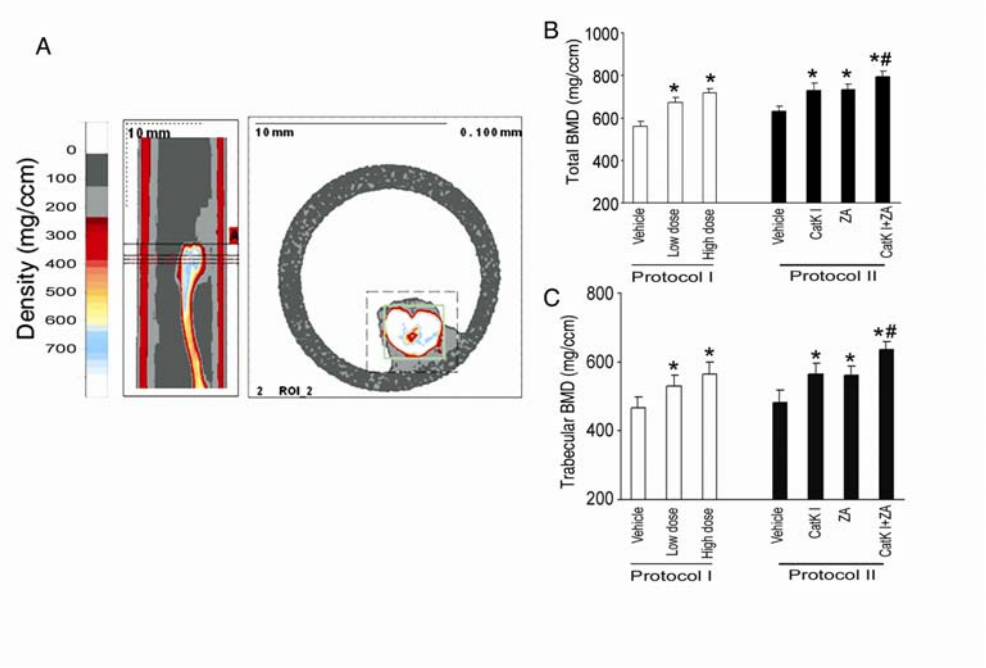


Figure 1B to 1E. CatK inhibitor prevented the PCa establishment and retarded progression of PCa tumor in mouse bone. (B) In this representative figure, note the area of osteolysis and osteosclerosis of the vehicle-treated mouse compared to the radiograph of the CatK inhibitor-treated mouse. PSA is strong positive in all vehicle-treated mice compared to the CatK inhibitor-treated mice. (C) Tumor volume vs non-bone soft tissue volume was measured by bone histomorphometry. (D) Serum PSA levels in the mice model were measured by ELISA. (E) Osteoclast numbers per millimeter bone surface were quantified by bone histomorphometry. Results are reported as mean ± SD. *P<0.01 compared to vehicle group; #P<0.01 compare to basal group.

CatK inhibitor treatment diminished tumor-induced loss of bone mineral density (BMD) based on pQCT analysis

To further confirm the alteration of tumor-induced bone lesions by approaches other than bone histomorphometric analysis, total and trabecular BMD for the tibiae were also measured by pQCT (Figure 2*A*).



CatK inhibitor treatment, both at low and high doses, significantly diminished the tumor-induced loss of total and trabecular BMD (Figure 2*B* and *C*). As expected, the CatK inhibitor also increased total and trabecular BMD in both protocols. The combination of the CatK inhibitor with ZA significantly enhanced both total BMD and trabecular BMD compared to any of the single agent alone (Figure 2*B* and *C*).

Figure 2. CatK inhibitor protected bone mineral density in a mouse model. (A) Slices that were scanned by pQCT. The small box in the right panel indicates the tibia. The reference slice and the slices examined were indicated on a representative tibia. (B) Total bone mineral density detected by pQCT of tibiae from each group. (C) Trabecular bone mineral density detected by pQCT of tibiae from each group. *P<0.01 compared to vehicle group; #P<0.01 compare to basal group.

CatK inhibitor had no effect on tumor growth at subcutaneous sites

As a parallel study for protocol 1, at the same time as the intratibial injection, C4-2B cells were injected into the subcutaneous sites. Subcutaneous tumor growth was measured for 8 weeks. Interestingly, we found CatK inhibitor had no direct effect on the tumor volume (Figure 3*A*) and the tumor cell proliferation *in vivo* (Figure 3*B* and *C*). These results suggest that the effect of CatK inhibitor maybe specific to the bone microenvironment.

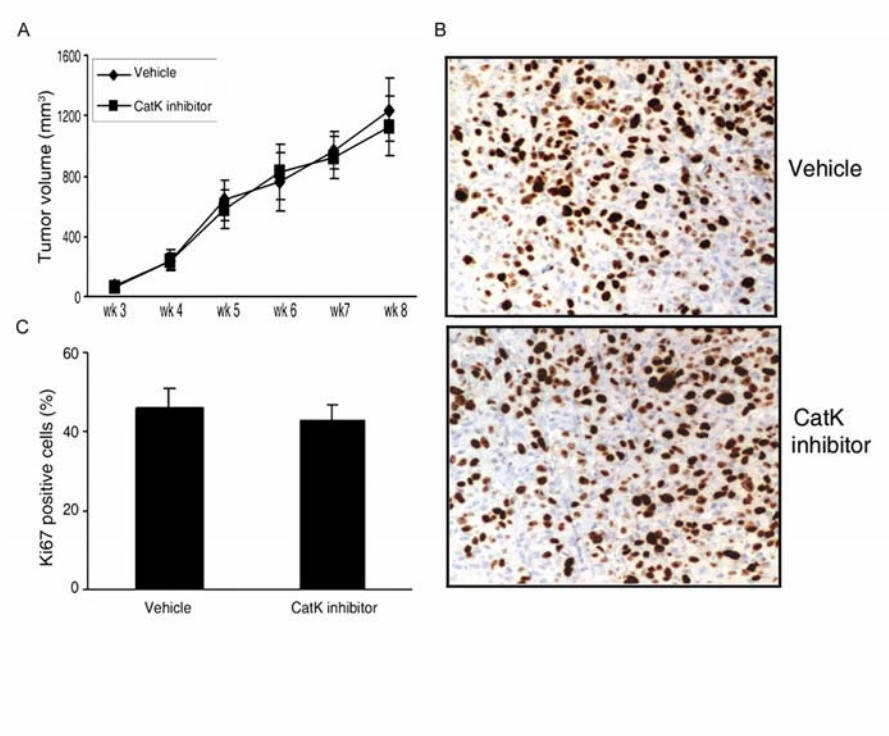


Figure 3. CatK inhibitor had no effect on subcutaneous tumor growth *in vivo*. (A) Tumor volume were monitored twice weekly by caliper measurements. (B) Subcutaneous tumor sections were immunohistochemically stained with Ki67 antibody. (C) Quantified data were determined by the number

of Ki67 positive cells dividing the total numbers in five randomly selected fields under light microscopy (× 400).

Therefore, we showed that the CatK inhibitor significantly reduced skeletal tumor burden as determined by both tumor volume vs soft tissue volume ratio and serum PSA levels. In contrast, ZA less effectively diminished the skeletal tumor volume. ZA failed to decrease PSA levels. Importantly, combinations of the two agents were more effective in decreasing tumor volume than any single agent. **Taken together, we show for the first time that a CatK inhibitor diminishes prostate cancer growth in bone and the inhibitory effects are enhanced by combination with ZA.**

In addition, we have extended this study using another tumor cells line (PC3). The reason is that PC3 cells caused a predominantly osteolytic lesion. As preliminary results, we found the very similar finding that combinations of the two agents were more effective in decreasing tumor volume than any single agent. In addition, the direct injection of tumor cells into the tibia has the advantage of nearly a 100% take rate, as opposed to a 20-30% take rate using the vascular approach (cardiac-injection). But we are aware that direct injection has the disadvantage of not modeling the entire metastatic cascade. Therefore, in PC3 cells, we have used cardiac-injection model. We will continue to collect the data and will apply for additional funds from DOD or other resources to get these experiments performed.

Finally, during this grant period, we unexpectedly found a critical chemokine, monocyte chemotactic protein-1 (MCP-1/CCL2), plays a key role in prostate cancer skeletal metastasis. We have generated excellent preliminary data in this research area, and published several peer-reviewed publications.

Key Research Accomplishments

1. We showed that the CatK inhibitor significantly reduced skeletal tumor burden as determined by both tumor volume vs soft tissue volume ratio and serum PSA levels.
2. In contrast, ZA less effectively diminished the skeletal tumor volume. ZA failed to decrease PSA levels.
3. Importantly, combinations of the two agents were more effective in decreasing tumor volume than any single agent, although these results should be determined in different cell lines and xenografts.
4. We have found one key factor, monocyte chemotactic protein-1 (MCP-1/CCL2), plays a key role in prostate cancer skeletal metastasis. We will apply for additional funds to continue this study and for now, we have generated several peer-reviewed publications.

Reportable Outcomes

1. An abstract, based on the up-to-date results, was selected as an oral presentation in Annual AACR meeting in Denver in April 2009 (Please see appendix 1) and the manuscript was submitted to the journal *Neoplasia* (Please see appendix 2).

2. Peer-reviewed publications with support of this DOD grant:

Lu Y, Chen Q, Yao Z, Keller ET, Dai J, Escara-Wilke J, Corey E, Zimmermann J, Zhang J*. Targeting cathepsin K diminishes prostate cancer establishment and growth in murine bone. Submitted to Neoplasia, 2010 (In revision)

Zhang J, Lu Y, and Pienta KJ. Multiple roles of CC chemokine ligand 2 (CCL2) in promoting prostate cancer growth. J Natl Cancer Inst. 2010 Apr 21;102(8):522-8.

Lu Y, Chen QY, Corey E, Xie W, Fan J, Dai J, Mizokami A, and Zhang J*. Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone. Clin Exp Metastasis, 26(2):161-9, 2009

Cai Z, Chen QY, Chen J, Lu Y, Xiao GZ, and Zhang J*. MCP-1 promotes lung cancer-induced bone resorptive lesions in vivo. Neoplasia, 11(3):228-236, 2009

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Lu Y, Cai Z, Xiao G, Keller ET, Mizokami A, Yao Z, Roodman GD, Zhang J*. Monocyte chemotactic protein-1 (MCP-1) mediates prostate cancer-induced bone resorption. Cancer Res, 67:3646-53, 2007

Lu Y, Xiao G, Galson DL, Nishio Y, Mizokami A, Yao Z, and Zhang J*. PTHrP-induced MCP-1 production by human bone marrow endothelial cells promotes osteosteoclastast differentiation and prostate cancer cell proliferation and invasion in vitro. Int J of Cancer. 121:724-733, 2007

Conclusion

From our studies, we showed that the cathesin K inhibitor diminishes prostate cancer growth in bone and the inhibitory effects are enhanced by combination with zoledronic acid.

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Yi Lu, Qiuyan Chen, Eva Corey, Jinlu Dai, June Escara-Wilke, Johann Zimmermann, Evan T. Keller, Jian Zhang. A cathesin K inhibitor diminishes prostate cancer growth in bone and the inhibitory effects are enhanced by combination with zoledronic acid. 2009 Denver AACR Proceedings abstract #2075

Targeting cathepsin K diminishes prostate cancer establishment and growth in murine bone. Yi Lu, Qiuyan Chen, Zhi Yao, Evan T. Keller, Jinlu Dai, June Escara-Wilke, Eva Corey, Johann Zimmermann, Jian Zhang *Neoplasia* (submitted)

Appendices

Please see the attached copy of AACR abstract and the manuscript.

Supporting data:

NA

Abstract Number: 2075

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Presentation Title: A cathesin K inhibitor diminishes prostate cancer growth in bone and the inhibitory effects are enhanced by combination with zoledronic acid

Presentation Start/End Time: Monday, Apr 20, 2009, 3:10 PM - 3:25 PM

Location: Room 405-407, Colorado Convention Center

Author Block: *Yi Lu*, *Qiuyan Chen*, *Eva Corey*, *Jinlu Dai*, *June Escara-Wilke*, *Johann Zimmermann*, *Evan T. Keller*, *Jian Zhang*. University of Pittsburgh, Pittsburgh, PA, University of Washington, Seattle, WA, University of Michigan, Ann Arbor, MI, Novartis Pharma Ltd., Basel, Switzerland

Prostate skeletal metastases are most often radiographically characterized as osteoblastic (increased bone mineral density) as opposed to osteolytic (decreased bone mineral density) lesions, it is clear that from histological evidence, however, prostate cancer skeletal metastases form a heterogeneous mixture of osteolytic and osteoblastic responses. Cathespin K (CatK) is a cysteine protease produced by osteoclasts. We and other researchers have shown that CatK is expressed not only by osteoclasts but also by prostate cancer cells and stromal cells. In addition, a highly selective CatK inhibitor has been shown to reduce the tumor-induced osteolysis and skeletal tumor burden in prostate and breast cancers in mouse models. Zoledronic acid (ZA), a bisphosphonate which exerts beneficial effects in prostate cancer patients with bone metastases, reduces both pain and skeletal related events. In this study, we hypothesized that combination of a bisphosphonate with the CatK inhibitor, through different inhibitory mechanisms, could diminish prostate cancer progression in vivo. Accordingly, human prostate cancer C4-2B cells were inoculated into the tibiae of SCID mice. The mice were randomized into the following treatment groups (n=10/group): ZA, CatK inhibitor, the combination of ZA and CatK inhibitor, and saline vehicle alone. ZA was injected s.c. twice weekly at 100ug/kg. CatK inhibitor was given at 50mg/kg by oral feeding 5 times per week. Test drugs were initiated at 4 weeks after the tumor cell inoculation and treatments were continued for 8 weeks. Histomorphometrical analysis, bone mineral density, and serum PSA measurement were used to determine the effects of treatment on the tumor growth in bone. We found that CatK inhibitor significantly reduced skeletal tumor burden as determined by both tumor volume vs soft tissue volume ratio and serum PSA levels. In contrast, ZA less effectively diminished the skeletal tumor volume. ZA failed to decrease PSA levels. Importantly, combinations of the two agents were more effective in decreasing tumor volume than any single agent. In a parallel study, C4-2B cells were injected subcutaneously into mouse flanks. The animals were given ZA, the CatK inhibitor, or vehicle (n=10). Subcutaneous tumor volume was monitored. Neither ZA nor CatK inhibitor inhibited the growth of sc tumors in animals suggesting the inhibitory effects of the two agents were bone-specific. Taken together, we show for the first time that a CatK inhibitor diminishes prostate cancer growth in bone and the inhibitory effects are enhanced by combination with ZA. This project is supported by DOD PC061231.

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Disclosure Information for CME-Designated Sessions

Targeting cathepsin K diminishes prostate cancer establishment and growth in murine bone[1]

Yi Lu[*, †, 2], Qiuyan Chen[*,‡, 2], Zhi Yao[†], Evan T. Keller[§], Jinlu Dai[§], June Escara-Wilke[§], Eva Corey[¶], Johann Zimmermann[#, 3], Jian Zhang[*]

Abbreviations: cathepsin K, CatK; osteoprotegerin, OPG; prostate cancer, PCa; parathyroid hormone-related protein, PTHrP; zoledronic acid, ZA; Receptor activator of nuclear factor KappaB ligand, RANKL

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Running Title: CatK inhibitor reduces prostate cancer growth in bone

Keywords: Cathepsin K, zoledronic acid, prostate cancer, skeletal metastasis, animal model

Abstract

The processes of prostate cancer (PCa) invasion and metastasis are facilitated by proteolytic cascade involving multiple proteases, such as matrix metalloproteinases, serine proteases and cysteine proteases including cathepsin K (CatK). CatK, degrades collagen I and expresses predominantly in osteoclasts, was recently demonstrated in breast cancer and PCa cells. Importantly, its expression level is greater in PCa bone metastatic sites compared to primary tumor and normal prostate tissues. The roles of CatK in PCa growth in bone, however, have not been studied. In this report, we first confirmed CatK expression in PCa LNCaP, C4-2B, and PC3 cells as well as in prostate cancer tissues. Second, we observed the inhibitory effects of a selective CatK inhibitor on PCa cell invasion. The CatK inhibitor dose-dependently inhibited PCa conditioned media-induced bone resorption. Third, we injected C4-2B cells into the tibiae of SCID mice. The animals received either vehicle or Cat K inhibitor for 8 weeks at the time of tumor cell injection (tumor establishment model; protocol 1) or 4 weeks after tumor cell injection (tumor progression model; protocol 2). Determined by radiograph, histology and bone histomorphometry, CatK inhibitor significantly prevented the tumor establishment in protocol 1, and reduced the tumor growth in bone in protocol 2. CatK inhibitor also decreased serum PSA levels in both animal models. Fourth, we demonstrated that the inhibitory effects of the CatK inhibitor were enhanced in combination with zoledronic acid (ZA). We conclude that the selective CatK inhibitor may be used as a novel therapeutic approach for advanced PCa.

Introduction

It is estimated that over 350,000 patients a year in the United States die with bone metastasis [1]. Prostate, breast, lung, and nanasopharyngeal carcinoma frequently metastasizes to skeletons, and among these patients, for example, skeletal metastases were identified in up to 90% of patients dying from prostate cancer (PCa) [2-5]. PCa skeletal metastases are most often radiographically characterized as osteoblastic (increased bone mineral density) as opposed to osteolytic (decreased bone mineral density) lesions, it is clear from histological evidence, however, PCa skeletal metastases form a heterogeneous mixture of osteolytic and osteoblastic responses [6-9]. Emerging evidence shows that osteoblastic metastases form on trabecular bone at sites of previous osteoclastic resorption, and that such resorption may be required for subsequent osteoblastic bone formation [10, 11]. The mechanisms through which PCa cells promote bone resorption and subsequent woven-bone formation remain poorly understood.

A number of reports have shown that osteoclast activities are important to the development of bone metastases in several cancer types including breast, lung, and PCa [12, 13]. As such, anti-resorptive approaches such as administration of bisphosphonates, i.e. zoledronic acid (ZA) or parathyroid hormone-related protein (PTHrP) neutralizing antibody have been reported in cancer animal models to block the tumor expansion in bone [14, 15]. In PCa skeletal metastasis animal models, we and other researchers have demonstrated antiresorptive agents such as soluble receptor activator of NFkB (sRANK) [16], osteoprotegerin (OPG) [11], and overexpression of OPG diminish the tumor growth in bone [17]. In clinics, although ZA effectively reduces metastasis-related bone pain and skeletal complications in patients with metastatic PCa and breast cancer [18-20], within a two-year period more than 30% of patients will experience at least one skeletal complication under the therapy with ZA [19-21]. Therefore, new treatments are urgently needed for patients who have bone metastases and those who are at high risk for developing bone metastases.

Cathepsin K (CatK), identified as an osteolytic and protease enzyme [22-25], degrades bone matrix proteins including type I collagen, osteopontin, and osteonectin [26, 27]. It has been revealed that CatK is

highly expressed in osteoclasts, but that cathepsins B, L and S are expressed at negligible levels [22, 23]. Patients with pycnodysostosis, a disease characterized by abnormal bone remodeling [28-30], have mutations in the *CatK* gene [31] and mice with a null mutation in the *CatK* gene develop osteopetrosis of the long bones and vertebrae [32]. Since CatK has one of the highest matrix degradation activities with higher efficiency than any other cathepsins and metalloproteinases (MMPs) [27, 33] and its ability to destroy matrix components, CatK and some of its family members have been implicated in diseases involving bone and cartilage destruction, including tumor invasion [34-36] and rheumatoid arthritis [37, 38].

CatK was reported in breast cancer cells that are PCaable of causing bone resorption [39] and CatK mRNA was also detected in PCa cell lines and in primary PCa and metastases [40]. Importantly, CatK expression in bone metastases was significantly greater than primary PCa, while CatK in normal prostate tissues was negative [40] suggesting that CatK may play a role in PCa skeletal metastases . Recently, a selective human CatK inhibitor has been described to potently inhibit osteoclast resorption both in vitro and in vivo [41-44]. In this study, we report that CatK contributes to PCa-induced osteoclast activity at bone metastatic sites, and inhibition of CatK by a selective inhibitor may prevent the establishment and progression of PCa in bone. We finally demonstrated that the inhibitory effects of the CatK inhibitor were enhanced in combination with zoledronic acid (ZA).

Materials and Methods

Cell Lines and Cell Culture

PCa PC3 and LNCaP cells were purchased from the American Type and culture Collection (ATCC, Manassas, VA) and were cultured in RPMI 1640 medium. C4-2B cells (UroCor, Oklahoma City, OK), derived from its parental LNCaP but with characteristics of skeletal metastasis, were maintained in T medium [80% DMEM, 20% Ham's F12 medium (Invitrogen, Carlsbad, CA), 5 µg/mL insulin, 13.6 pg/mL triiodothyronine, 5 µg/mL transferrin, 0.25 µg/mL biotin, and 25 µg/mL adenine (Sigma, St. Louis, MO)]. Primary murine bone marrow cells (MBMC) were cultured in αMEM medium. All cell cultures were supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) (HyClone, Pittsburgh, PA). Prostate epithelial cells (PrEC) are primary human epithelial cells (Cambrex, Walkersville, MD) and were maintained in PrEGM BulletKit media (Cambrex). All cells were maintained in a 37 °C incubator equilibrated with 5% CO_2.

Animals

Male SCID mice (Charles River, Wilmington, MA) at 6 weeks of age were housed under pathogen-free conditions in accordance with the NIH guidelines. The animal protocol was approved by the Institutional Animal Care and Use Committee, both from the University of Pittsburgh and the University of Michigan.

CatK Inhibitor

Cathepsin K selective inhibitor and zoledronic acid used in these studies was provided by Novartis Pharma Ltd., (Basel, Switzerland). The inhibitor dosages in this *in vivo* study were chosen as 50mg/kg/day and 100mg/kg/day based on the renal toxicity of dose 500mg/kg/day in rat and preclinical efficacy and tolerability dose of 50mg daily used in human.

Immunohistochemical Staining for CatK

Tissue microarray of PCa specimens (83 Asian patients who underwent radical prostatectomy with Gleason score 5-10: n=83) were purchased from IU Abxis (Seoul, Korea) with corresponding non-neoplastic tissues (42 specimens). Slides were heated at 55°C for 30 minutes, deparaffinized and rehydrated, then antigen retrieval was performed with Target Retrieval kit from Dako (Carpinteria, CA). Slides were incubated for 24 h at 4°C with goat anti-human CatK polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA; 1:200 dilution) , or isotype control goat IgG (Santa Cruz; 1:200 dilution). Biotinylated anti-goat (Dako) at a dilution of 1:500 was used as the secondary antibody. After incubation in avidin-biotin complex solution (Dako), the staining was developed by the diaminobenzidine method, followed by counterstaining with hematoxylin.

Obtaining Conditioned Media (CM)

CM were obtained from PCa cells as previously described [45]. Briefly, 2 x 10^6 cells were plated in 10-cm tissue culture dishes for 12 hours in RPMI 1640 with 10% FBS. The media were then changed to 10 ml of RPMI 1640 plus 1% FBS, and supernatants were collected 48 hours later. To normalize for differences in cell density because of proliferation during the culture period, cells from each plate were collected and total DNA content/plate was determined. CM were then normalized for DNA content between samples by adding RPMI.

CatK mRNA Expression and Quantification CatK mRNA

Total RNA was extracted from LNCaP, C4-2B, PC3 and PrEC cells using TRIzol reagent (Life Technologies, Gaithersburg, MD), then subjected to RT-PCR for detection Cat K mRNA. PCR primers used for detection of CatK consisted of sense 5'-CAG CAA AGG TGT GTA TTA TGA TGA AAG C-3', and antisense 5'-ATG GGT GGA GAG AAG CAA AGT AGG AAG G-3' resulting in a PCR product of 399 bp (Genebank accession no. X82153). Beta-actin cDNA was amplified as a control for RNA quality. The PCR products were subjected to eletrophoresis on a 1.5% agarose gel, stained with ethidium bromide. For quantification of CatK

mRNA expression, real-time RT-PCR was performed in an iCycler iQ multicolor real-time RT-PCR detection system (Bio-Rad, Hercules, CA) using the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad). Melting curve analysis was performed to evaluate purity of the PCR products. Triplicate samples were run for each primer set. The relative expression of CatK to GAPDH (as housekeeping gene control) was calculated using the ΔCT method as previously described [46]. CatK primers were: sense 5'-CAG CAG AGG TGT GTA CTA TG-3' and antisense 5'-GCG TTG TTC TTA TTC CGA GC-3'. GAPDH primers were: sense 5'-CCA TGG AGA AGG CTG GGG-3' and antisense 5'-CAA AGT TGT CAT GGA TGA CC-3'.

Immunoblot Analysis

To evaluate the CatK protein expression in PCa cells, confluent LNCaP, C4-2B, PC3 and PrEC cells were washed with PBS and lysed. Protein concentration of the cell lysates was measured by BCA kit (Pierce, Rockford, IL) and proteins (40μg/lane) were applied to SDS-PAGE followed by Western blot with goat anti-human CatK polyclonal Antibody (diluted 1:100 in blocking solution) (Santa Cruz Biotech, Santa Cruz, CA), or mouse anti-human β-actin monoclonal antibody (Sigma; diluted 1:2000 in blocking solution)). The antibody binding was revealed using an HRP-conjugated anti-goat IgG, or HRP-conjugated anti-mouse IgG1 (Santa Cruz) at 1:5000 and enhanced chemiluminescence (ECL) blot detection system (Amersham Biosciences, Piscataway, New Jersey).

ELISA

To evaluate CatK protein production by PCa cells, CM collected from LNCaP, C4-2B, PC3 and PrEC cell cultures were measured by CatK ELISA kit (Alpco, Windham, NH) following the manufacturer's protocol. Means ± standard errors were calculated from triplicates.

Knockdown Experiment by siRNA

The designed CatK siRNA, or scrambled siRNA was transfected into C4-2B cells using the transfection reagents from Santa Cruz. In brief, C4-2B cells were cultured in 6-well plates (3×10^5/well) with antibiotic-free cell growth medium 1 day prior to transfection. For each transfection, 1 µg of siRNA duplex was diluted in siRNA transfection medium to a final volume of 100 µl. Six microliter siRNA transfection reagent was diluted into 100 µl siRNA transfection medium and added directly into the siRNA duplex solution. After 30 min incubation at room temperature, 0.8 ml transfection medium was added to each tube containing the siRNA and transfection reagent mixture,

then overlayed the mixture onto the cells which had been washed by siRNA transfection medium. Cells were incubated for 6 hours at 37ºC and cell growth media containing 2x serum and antibiotics were then added. The cells were incubated for an additional 24 hours and the media was replaced with cell growth media for 48 hours. Cell lysates were collected for western blot to confirm CatK expression was knockdown.

Cell Proliferation

Cell proliferation was measured by a CellTiter 96 AQeous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, C4-2B cells were plated in 96-well plates at a density of 5000 cells/well in 200 µL of T medium with 5% FBS. After 12 hours of culture, the media was changed to RPMI 1640 plus 0.5% FBS and a different concentration (0-100µM) of CatK inhibitor was added. The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 hours, then 20 µL of MTS/PMS solution was added. After incubation for 2 hours at 37 °C, the absorbance of each well at 490 nm was recorded by using an ELISA plate reader. Data represent the average absorbance of six wells in one experiment.

In Vitro Cell Invasion Assay

The *in vitro* invasion assay was performed using C4-2B cells in the presence or absence of CatK inhibitor and C4-2B cells transfected with either CatK siRNA or control siRNA. The invasiveness of cells was

evaluated in 24-well matrigel invision chamber (BD Biosciences, Bedford, MA), as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes (8-µm pore size). The membranes in some wells were pre-coated with 100 µg/cm^2 of collagen matrix (Matrigel) that was reconstituted by adding 0.5 ml of serum-free T medium to the well for 2 hours. To assess the ability of the cells to cross the polycarbonate membrane (i.e., baseline migration), 2.5 x 10^4 cells in 0.5 ml of T medium containing 5% FBS was placed into the upper compartment of wells that did not contain collagen matrix, and 0.75 ml of T medium containing 10% FBS was placed into the lower compartment. In parallel, we assessed the ability of the same cells to penetrate a collagen matrix by placing 2.5 x 10^4 cells in 0.5 ml of T medium containing 5% FBS in the upper compartment of wells that were coated with the reconstituted matrix and 0.75 ml of T medium containing 10% FBS. In upper compartments, different concentration of CatK inhibitor (0, 1, 10, 50, 100 µM) was added. The transwell chambers were incubated for 24 hours at 37 °C in 95% air and 5% CO_2. Cell penetration through the membrane was detected by staining the cells on the porous membrane with a Diff-Quik stain kit (Dade Behring, Newark, DE) and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at x200 magnification) per filter. Invasive ability was defined as the proportion of cells that penetrated the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of triplicate assays.

In Vitro Bone Resorption Assay

CM (25%, v/v) from C4-2B cells was added to non-adherent primary murine bone marrow cells (1x 10^5 from C57BL/6 mouse femurs) that seeded into the 24-wells of BD Biocoat Osteologic Bone cell culture system (BD Bioscience) that consist of sub-micro synthetic calcium phosphate thin films coated on to the culture vessels and on dentin slices in 96-wells plate. Soluble RANKL (50ng/ml) and MCSF (10ng/ml) were used as positive controls or different concentration of the CatK inhibitor was added. The osteoclast culture was

maintained for 10 days and half of the media was changed every 3 days. Then the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) (Kamiya Biomedical, Seattle, WA). Resorptive area on the digital images of osteologic or dentin slices were measured with a BIOQUANT system (R&M Biometrics, Inc., Nashville, TN). Samples were evaluated in triplicates.

Experimental Protocols for Intraosseous Tumorigenesis

The animal studies are summarized in figure 4A. In protocol 1, mice were randomized to receive either vehicle or CatK inhibitor (five times per week, 50mg/kg/day as low dose and 100mg/kg/day as high dose, orally, n=10) at the time of C4-2B cell injection (3×10^5 cells) and continued for 8 weeks. In protocol 2, four weeks after the tumor cells injection, the mice were randomized into the following treatment groups (n=10/group): ZA, CatK inhibitor, the combination of ZA and CatK inhibitor, and saline vehicle alone. ZA was injected s.c. once every 4 weeks at 100ug/kg. CatK inhibitor was given at 50mg/kg. Test drugs were given for 8 weeks. One group was sacrificed as basal control. Before sacrifice, the animals were anaesthetized, and magnified flat radiographs were taken with a Faxitron (Faxitron X-Ray Corp, Wheeling, Illinois). At sacrifice, the blood was collected and measured for PSA levels. All of the major organs and lumbar vertebrae were harvested for histological analysis.

Histopathology and Bone Histomorphometry

Histopathology was performed as described previously [16]. Briefly, bone specimens were fixed in 10% formalin and decalcified in 10% EDTA for 6 days. The specimens were then paraffin embedded, sectioned (5 μM), and stained with hematoxylin and eosin (H&E). Nonstained sections were deparaffinized and rehydrated then stained for prostate-specific antigen (PSA) with anti-human PSA antibody using standard immunohistochemistry techniques. To perform TRAP staining, nonstained sections were stained for TRAP. For routine histopathology, soft tissues were preserved in 10% formalin, embedded in paraffin, sectioned (5 μM),

and stained with H&E. To evaluate xenografts proliferation, sections were deparaffinized, rehydrated and stained with Ki67 monoclone antibody following a modified protocol [47]. The Ki-67 labeling index (KI) was calculated as the percentage of positive tumor nuclei divided by the total number of tumor cells examined. At least 1,000 tumor cells per specimen were examined in five randomly selected fields by light microscopy (x400) by an investigator who was blinded to the animal groups as we have previously described [48]. Histomorphometric analysis was performed on a BIOQUANT system (BIOQUANT-R&M Biometrics Inc) as described previously [16]. Four discontinuous random regions of interest were examined within each tibia, without knowledge of treatment group, by examination at ×100. Tumor volume was determined as the proportion of tumor area in the total nonmineralized portion of the bone.

Bone Mineral Density Analysis

Total bone mineral density and trabecular bone mineral density of tibiae were measured using peripheral quantitative computed tomography (pQCT) (Stratec, Ontario, CA). Scans from three slices of the proximal metaphases region were performed to obtain trabecular bone mineral densities.

Subcutaneous Tumors

As a parallel study for protocol 1, at the same time as the intratibial injection, C4-2B cells were resuspended in T media. Two million cells were mixed 1:1 with Matrigel (Collaborative Biomedical Products, Bedford, Massachusetts), and then injected into the right flank at 100 μl/site using a 23-g needle (n=10/group). Subcutaneous tumor growth was monitored by palpation, and two perpendicular axes were measured; the tumor volume was calculated using the formula as: volume = length × $width^2/2$.

PSA Measurement

Total PSA levels in serum were determined using the Accucyte Human PSA assay (Cytimmune Sciences, College Park, MA). The sensitivity of this assay is 0.488 ng/ml.

Statistical Analysis

Statistical analysis was performed using Statview software (Abacus Concepts, Berkley, CA). For comparing two groups, Student's t test was used. ANOVA was used for initial analyses of comparing multiple groups, followed by Fisher's protected least significant difference for *post hoc* analyses. Differences with a $P < 0.05$ were determined as statistically significant.

Results

CatK is Highly Expressed in aggressive PCa Cells

Brubaker et al. reported that CatK protein were positively stained in up to 40% of primary PCa and nearly 100% of metastatic PCa cells at skeletal sites in human samples, whereas normal prostate glands were negative [40]. To confirm CatK expression in PCa, we performed immunohistochemistry on a tissue microarray that was consisted of 83 specimens from PCa patients with corresponding non-neoplastic prostate tissues. As a representative staining slide (figure 1*A*), CatK positive staining was at non- or minimal level (13%) in the non-neoplastic prostate glands, but at higher level (82%) in aggressive PCa. These results were consistence with those previously reported by Brubaker et al. [40]. To test whether CatK protein was expressed in PCa cells, total RNA and cell lysates were collected from PCa LNCaP, C4-2B, PC3 cells and normal prostate PrEC cells. All PCa LNCaP, C4-2B, and PC3 cells expressed higher levels of CatK mRNA than that of PrEC cells (Figure 2*B*-*C*). More aggressive C4-2B and PC3 cells expressed greater levels of both CatK mRNA and protein than the less aggressive LNCaP cells (Figure 1*B*-*D*). Cat K was present in the CM collected from all the PCa cells determined by ELISA (Figure 1*E*). C4-2B and PC3 cells produced greater levels of CatK protein than LNCaP cells (Figure 1*E*). In addition, CatK enzyme activity was also detected in LNCaP, C4-2B, and PC3 cells using an assay for degradation of collagen I as previously described (data not shown) [40].

CatK Inhibitor Diminishes PCa Tumor Invasion in vitro

To test the possible mechanism of tumor growth inhibition by CatK inhibitor in bone, the *in vitro* cell invasion assay was performed. We observed that CatK inhibitor diminished invasiveness of C4-2B cells *in vitro* in a dose-dependent fashion (Figure 2*A*). We examined the C4-2B cell viability using the cells treated with various doses of CatK inhibitor. We did not observe any significant differences among these cells (Figure 2*B*) suggesting that the CatK inhibitor had no toxicity for the tumor cells in our chosen doses. To further

confirm the role of CatK in the tumor cell invasion, we used C4-2B cells in which CatK expression was knocked down by siRNA and scrambled siRNA control cells to measure the ability of cell invasion and proliferation. CatK protein expression was significantly (95%) knocked down (Figure 2C) and the CatK siRNA knockdown, as expected, inhibited C4-2B cell invasion but did not affect the cell proliferation (Figure 2*C-E*). This result suggests that blocking the tumor cell invasiveness by CatK inhibitor could be a novel mechanism of CatK's function in PCa progression.

CatK Inhibitor Diminishes PCa-CM-Induced Bone Resorption in vitro

To evaluate the ability of the CatK inhibitor to diminish the PCa-CM-induced bone resorption, we first collected CM from C4-2B cell culture as we previously published [11]. We seeded non-adherent primary murine bone marrow cells into the 24-wells of BD osteologic bone cell culture system that consist of sub-micro synthetic calcium phosphate thin films coated onto the culture vessels. As a parallel study, a 96-well plate which contained dentin slice was also tested for bone resorption assay. Soluble RANKL (50ng/ml as a positive control) or the indicated concentration of CatK inhibitor was added. We observed that C4-2B CM-induced bone resorption in both systems (Figure 3*A* and *B*) and this induction was significantly diminished by the CatK inhibitor in a dose-dependent manner when we quantified bone resorption area on the dentin slides (Figure *B*).

CatK Inhibitor Prevents Establishment and Retards Progression of PCa Tumor in Murine Bone

To determine if inhibition of osteoclastogenic activity by the CatK inhibitor could prevent establishment or retard progression of PCa *in vivo*, we directly injected C4-2B cells into the tibia of SCID mice. The experimental protocols are summarized in figure 4*A*. Additionally, we injected C4-2B cells into subcutaneous sites of the same mice in protocol 1 to evaluate differences in response in the bone versus a non-osseous site. In protocol 1, the mice were administered orally either CatK inhibitor or vehicle either at the time of tumor cell injection. In protocol 2, four weeks after the tumor cells injection, the mice were randomized into groups: ZA,

CatK inhibitor, the combination of ZA and CatK inhibitor, and saline vehicle alone. Test drugs were given for 8 weeks. We found that CatK inhibitor significantly inhibited the establishment and development of PCa tumor in bone (Figure 4*B* and *C*). C4-2B caused dominantly osteolytic areas with some areas of osteoblastic activity (Figure 4*B*). It significantly reduced tumor volume (Figure 4*C*), serum PSA levels (Figure 4*D*) and osteoclast formation at bone-tumor interface (Figure 4*E*) compared to the vehicle-treated animals. These results indicated tumor burden was reduced by administration of the CatK inhibitor. In contrast, ZA less effectively diminished the skeletal tumor volume compared to the administration of the CatK inhibitor (Figure 4*C*). Interestingly, ZA failed to decrease PSA levels in these animals (Figure 4*D*). The inhibitory effects of the CatK inhibitor were enhanced in combination with zoledronic acid treatment (Figure 4*B* to 4*D*).

CatK inhibitor treatment diminished tumor-induced loss of bone mineral density (BMD) based on pQCT analysis

To further confirm the alteration of tumor-induced bone lesions by approaches other than bone histomorphometric analysis, total and trabecular BMD for the tibiae were also measured by pQCT (Figure 5*A*). CatK inhibitor treatment, both at low and high doses, significantly diminished the tumor-induced loss of total and trabecular BMD (Figure 5*B* and *C*). As expected, the CatK inhibitor also increased total and trabecular BMD in both protocols. The combination of the CatK inhibitor with ZA significantly enhanced both total BMD and trabecular BMD compared to any of the single agent alone (Figure 5*B* and *C*).

CatK inhibitor had no effect on tumor growth at subcutaneous sites

As a parallel study for protocol 1, at the same time as the intratibial injection, C4-2B cells were injected into the subcutaneous sites. Subcutaneous tumor growth was measured for 8 weeks. Interestingly, we found CatK inhibitor had no direct effect on the tumor volume (Figure 6*A*) and the tumor cell proliferation *in vivo*

(Figure 6*B* and *C*). These results suggest that the effect of CatK inhibitor maybe specific to the bone microenvironment.

Discussion

Prostate cancer skeletal metastases cause significant complications including severe bone pain, impaired mobility, pathological fracture, spinal cord compression and hypercalcemia [49, 50]. In spite of the radiographic osteoblastic appearance it is clear from histological evidence that PCa metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions although osteoblastic lesions are predominate [6-9]. Recent evidence shows that osteoblastic metastases form on trabecular bone at sites of previous osteoclastic resorption, and that such resorption may be required for subsequent osteoblastic bone formation [10, 11].

Cysteine proteases have been implicated in the cancer progression, including tumor cell aberrant proliferation and apoptosis, angiogenesis, invasion, and metastasis. This suggests that cysteine proteases are relevant drug targets for treating cancer [51]. In addition, cysteine cathepsins' upregulation has been reported for different types of human tumors, including breast, lung, brain, gastrointestinal, PCa, and melanoma [52]. CatK, a key component in bone remodeling, was recently identified with the highest matrix degradation activities. It also has higher efficiency than any other cathepsins and metalloproteinases (MMPs) [27, 33]. CatK knockout mice showed an osteopetrotic phenotype associated with a severe impairment of resorptive activity of osteoclasts [53]. Several lines of evidence have shown that osteoclastic lesions are important to the development of bone metastases [12]. Anti-resorptive approaches such as administration of bisphosphonates or anti-PTHrP neutralizing antibody have been reported in breast cancer models to be able to block the tumor expansion in bone [14, 15]. Bisphosphonates could prevent bone loss by inducing cell death of osteoclasts. However, bisphosphonates have several disadvantages, such as upper gastrointestinal irritation, fever, pain, and delayed fracture healing and have a very long half-life in human body (>10 years), which is a serious concern [54]. Furthermore, it has been recently reported that that bisphosphonates are PCaable of causing osteonecrosis of the jaws [55-57] and bisphosphonates was shown to induce inflammation and rupture of atherosclerotic plaques in apolipoprotein-E null mice [58]. Therefore new treatments are needed. Our findings in the current study show that a selective CatK inhibitor could provide an alternative approach and support a recent report that

showed using CatK inhibitors would be more beneficial than using bisphosphonates [59] because they have a positive effect on bone formation in addition to the inhibition of bone resorption.

In our experiments, we have found prostate cancer cell lines, C4-2B, LNCaP, and PC3 expressed CatK mRNA and protein, and prostate epithelial cells, PrEC expressed very low CatK. CatK's expressions were higher in metastatic prostate cancer cells—C4-2B and PC3 cells than in nonmetastatic cells—LNCaP cells either in mRNA level or protein level. We also found that CatK were expressed at low level in the normal prostate, increased in primary prostate tumor, and at high level in a skeletal metastatic tumor by immunohistochemistry of tissue samples (tissue microarray). Therefore, these is a possibility that the selective CatK inhibitor may target not only the bone cells, but also the tumor cells.

CatK inhibitor using in this study is an enzyme inhibitor which should not adversely affect the tumor cell and should leave the interplay between bone resorbing and bone forming cells intact. Accordingly, we examined the C4-2B cells viability that treated with indicated doses of CatK inhibitor. We didn't observe any significant differences. Interestingly, CatK inhibitor diminished invasion of C4-2B cells *in vitro*. Using a CatK siRNA technique, we obtained similar results in regard to tumor cell invasion as using a CatK inhibitor. Furthermore, we found that the CatK inhibitor dose-dependently diminished PCa CM-induced bone resorption *in vitro*. These results indicate that CatK may play an important role in both PCa progression and bony metastasis.

To further determine the effects of CatK inhibitor *in vivo*, we observed CatK inhibitor could prevent establishment of PCa tumor in mouse bone by using tumor establishment model, and retard tumor progression in murine bone by using tumor progression model. As expected, the CatK inhibitor increased bone mineral density in both mouse models. Critically, we showed, for the first time, that the inhibitory effects of the CatK inhibitor were enhanced in combination with ZA. These results provide a rationale to use the CatK inhibitor in combination with ZA in order to achieve better clinical outcomes for the treatment.

Taken together, we show that CatK inhibitor reduces prostate cancer-induced bone lesions, diminishes tumor burden in bone, and increases bone formation. Our data suggest that CatK inhibitor is a potential agent for the treatment of bone metastases. This novel and unique targeting strategy has established the rationale to inhibit tumor-induced bone resorption at bone metastatic sites.

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Figure legends

Figure 1. CatK mRNA and protein expression in PCa tissues and cell lines. (A) CatK expression in human PCa tissues. Immunohistochemical staining was performed for detection of CatK in human PCa and corresponding non-neoplastic tissues. (B) Total RNA was extracted from prostate epithelial cells (PrEC), LNCaP, C4-2B, and PC3 cells, and subjected to RT-PCR for detection CatK mRNA. PCR product of 399 bp was detected. (C) Quantification of CatK mRNA was determined by real-time PCR. Internal control was β-actin. *P<0.05 compared to PrEC cells; #P<0.01 compared to LNCaP cells. (D) Prosteins were collected from PrEC, LNCaP, C4-2B, and PC3 cells and subjected to Western blot. (E) CM collected from PrEC, LNCaP, C4-2B, and PC3 cell cultures were measured by an ELISA kit. *P<0.01 compared to PrEC cells; #P<0.01 compared to LNCaP cells.

Figure 2. CatK inhibitor diminished the invasiveness of C4-2B cells. (A) The *in vitro* invasion assay was performed using C4-2B cells cultured in a transwell chamber. Cell penetration through the membrane was detected by staining the cells on the porous membrane with a Diff-Quik stain kit and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at ×200 magnification) per filter. Invasive ability was defined as the proportion of cells that penetrated the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of triplicate assays. *P<0.05 compared to the vehicle; #P<0.001 compared to the vehicle. (B) The C4-2B cells viability were examined by MTS assay. C4-2B cells were treated with various doses of CatK inhibitor (the doses that were used in the tumor invasion assay). (C) The designed CatK siRNA, or scrambled control siRNA were transfected into C4-2B cells. Cell lysates were collected for Western blot to confirm CatK expression was knocked down. (D) Cell viability was measured by MTS assay. (E) The cell invasion was measured for the CatK knockdown cells and compared to the control cells. The results are reported as the mean of triplicate assays. *p < 0.01 compared to the control siRNA-transfected cells.

Figure 3. CatK inhibitor diminishes PCa-CM-induced bone resorption *in vitro*. (A) Representative images of resorption pits on dentin slices or synthetic calcium phosphate thin films are shown. Left panels without a frame: BD Biocate osteologic bone cell culture system, right panels with a frame: pit assay. (B) Samples were evaluated in triplicates and the resorption area was quantified. Results are reported as mean ±SD. *P<0.001 compared to Non-treatment (Non-TX) group; #P<0.01 compared to C4-2B CM-treated group; **P<0.001 compared to C4-2B CM-treated group.

Figure 4. CatK inhibitor prevented the PCa establishment and retarded progression of PCa tumor in mouse bone. (A) Schematic of experimental procedures to determine the effects of CatK inhibitor on the establishment and progression of prostate cancer. (B) In this representative figure, note the area of osteolysis and osteosclerosis of the vehicle-treated mouse compared to the radiograph of the CatK inhibitor-treated mouse. PSA is strong positive in all vehicle-treated mice compared to the CatK inhibitor-treated mice. (C) Tumor volume vs non-bone soft tissue volume was measured by bone histomorphometry. (D) Serum PSA levels in the mice model were measured by ELISA. (E) Osteoclast numbers per millimeter bone surface were quantified by bone histomorphometry. Results are reported as mean ± SD. *P<0.01 compared to vehicle group; #P<0.01 compare to basal group.

Figure 5. CatK inhibitor protected bone mineral density in a mouse model. (A) Slices that were scanned by pQCT. The small box in the right panel indicates the tibia. The reference slice and the slices examined were indicated on a representative tibia. (B) Total bone mineral density detected by pQCT of tibiae from each group. (C) Trabecular bone mineral density detected by pQCT of tibiae from each group. *P<0.01 compared to vehicle group; #P<0.01 compare to basal group.

Figure 6. CatK inhibitor had no effect on subcutaneous tumor growth *in vivo*. (A) Tumor volume were monitored twice weekly by caliper measurements. (B) Subcutaneous tumor sections were immunohistochemically stained with Ki67 antibody. (C) Quantified data were determined by the number of Ki67 positive cells dividing the total numbers in five randomly selected fields under light microscopy (× 400).

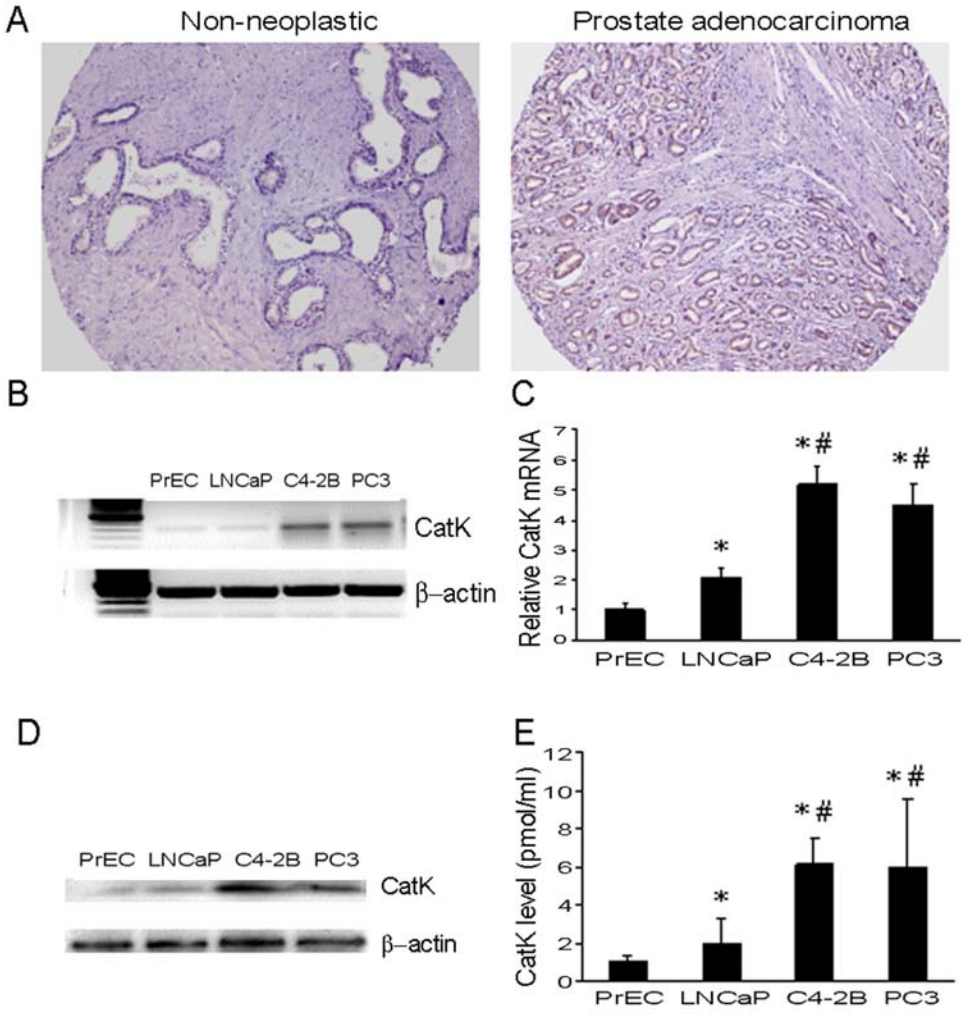
A
Non-neoplastic
Prostate adenocarcinoma
B
PrEC LNCaP C4-2B PC3
CatK
β–actin
C
Relative CatK mRNA
0 1 2 3 4 5 6 7
*
*#
*#
PrEC LNCaP C4-2B PC3
D
PrEC LNCaP C4-2B PC3
CatK
β–actin
E
CatK level (pmol/ml)
0 2 4 6 8 10 12
*
*#
*#
PrEC LNCaP C4-2B PC3

Figure 1

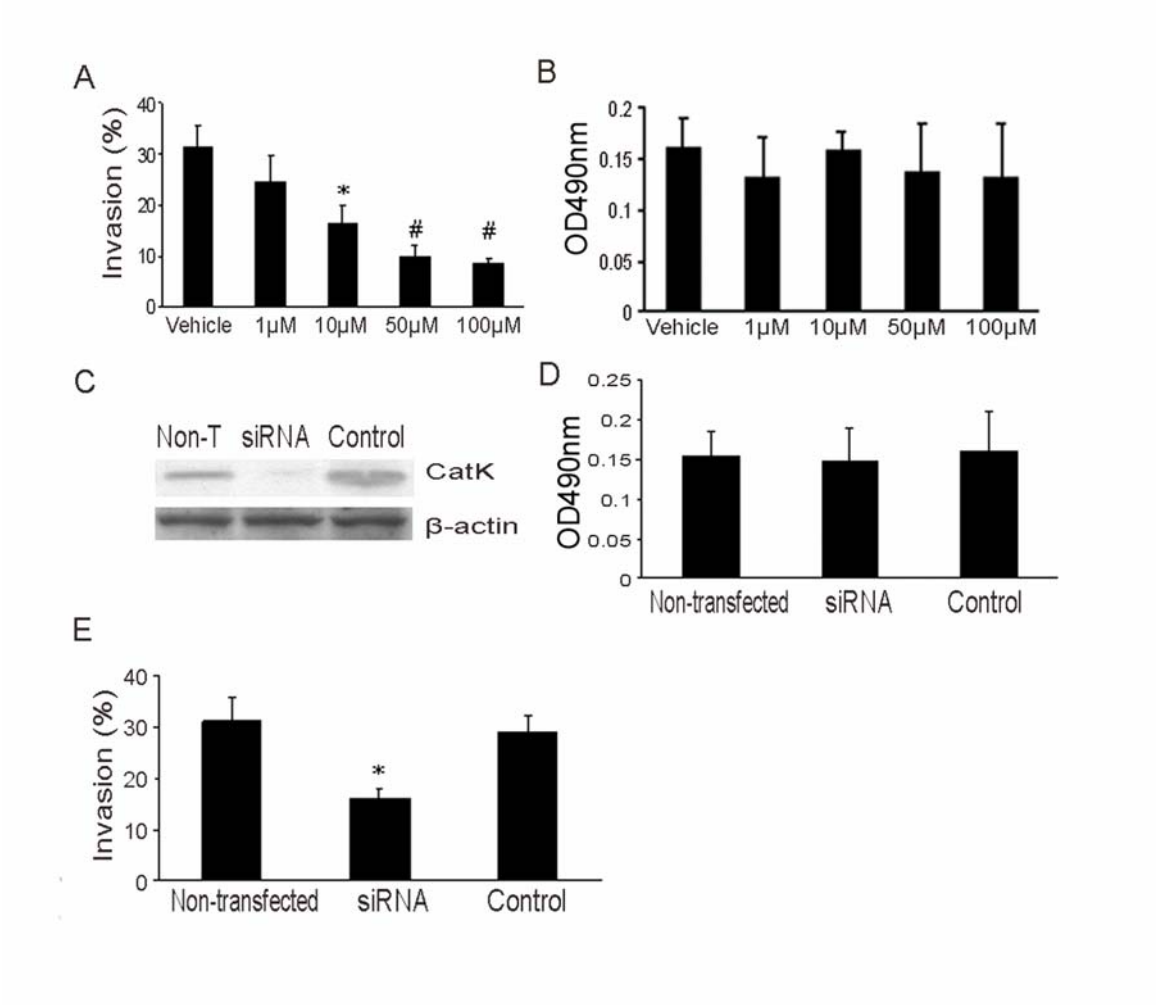
A
Invasion (%)
40
30
20
10
0
*
#
#
Vehicle
1μM
10μM
50μM
100μM
B
OD490nm
0.2
0.15
0.1
0.05
0
Vehicle
1μM
10μM
50μM
100μM
C
Non-T
siRNA
Control
CatK
β-actin
D
OD490nm
0.25
0.2
0.15
0.1
0.05
0
Non-transfected
siRNA
Control
E
Invasion (%)
40
30
20
10
0
*
Non-transfected
siRNA
Control

Figure 2

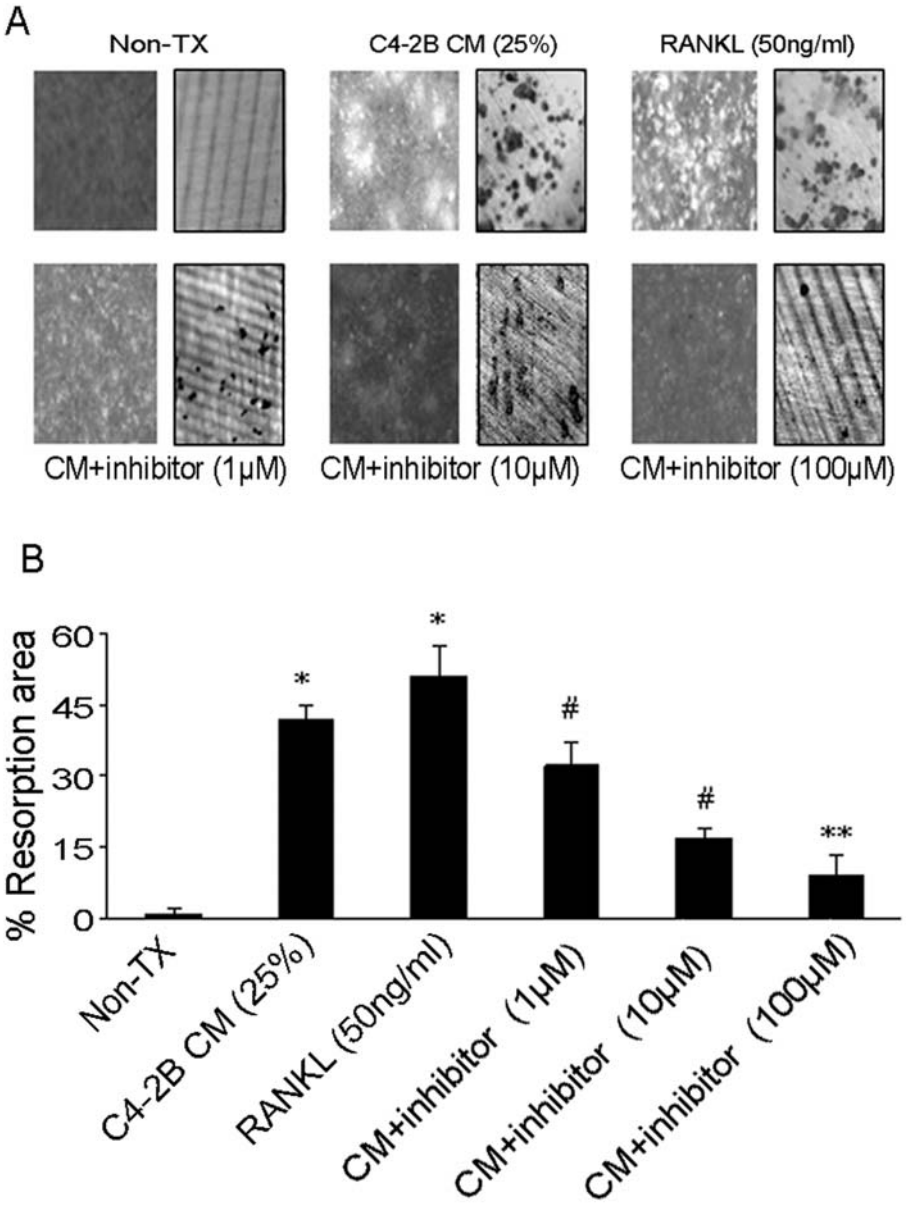
A
Non-TX
C4-2B CM (25%)
RANKL (50ng/ml)
CM+inhibitor (1μM)
CM+inhibitor (10μM)
CM+inhibitor (100μM)
B
% Resorption area
60
45
30
15
0
*
*
#
#
**
Non-TX
C4-2B CM (25%)
RANKL (50ng/ml)
CM+inhibitor (1μM)
CM+inhibitor (10μM)
CM+inhibitor (100μM)

Figure 3

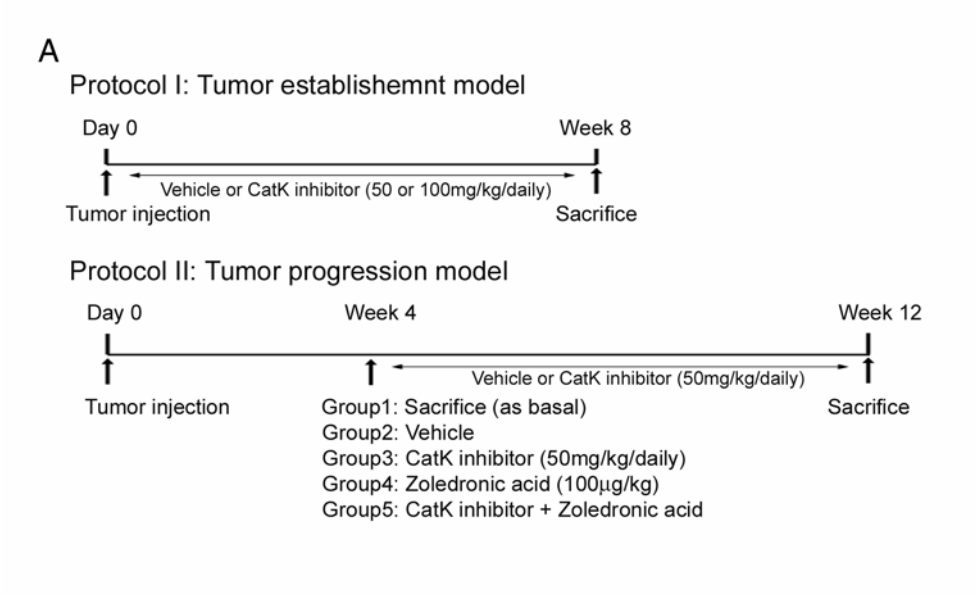
A
Protocol I: Tumor establishemnt model
Day 0
Week 8
Vehicle or CatK inhibitor (50 or 100mg/kg/daily)
Tumor injection
Sacrifice
Protocol II: Tumor progression model
Day 0
Week 4
Week 12
Vehicle or CatK inhibitor (50mg/kg/daily)
Tumor injection
Group1: Sacrifice (as basal)
Group2: Vehicle
Group3: CatK inhibitor (50mg/kg/daily)
Group4: Zoledronic acid (100μg/kg)
Group5: CatK inhibitor + Zoledronic acid
Sacrifice

Figure 4A

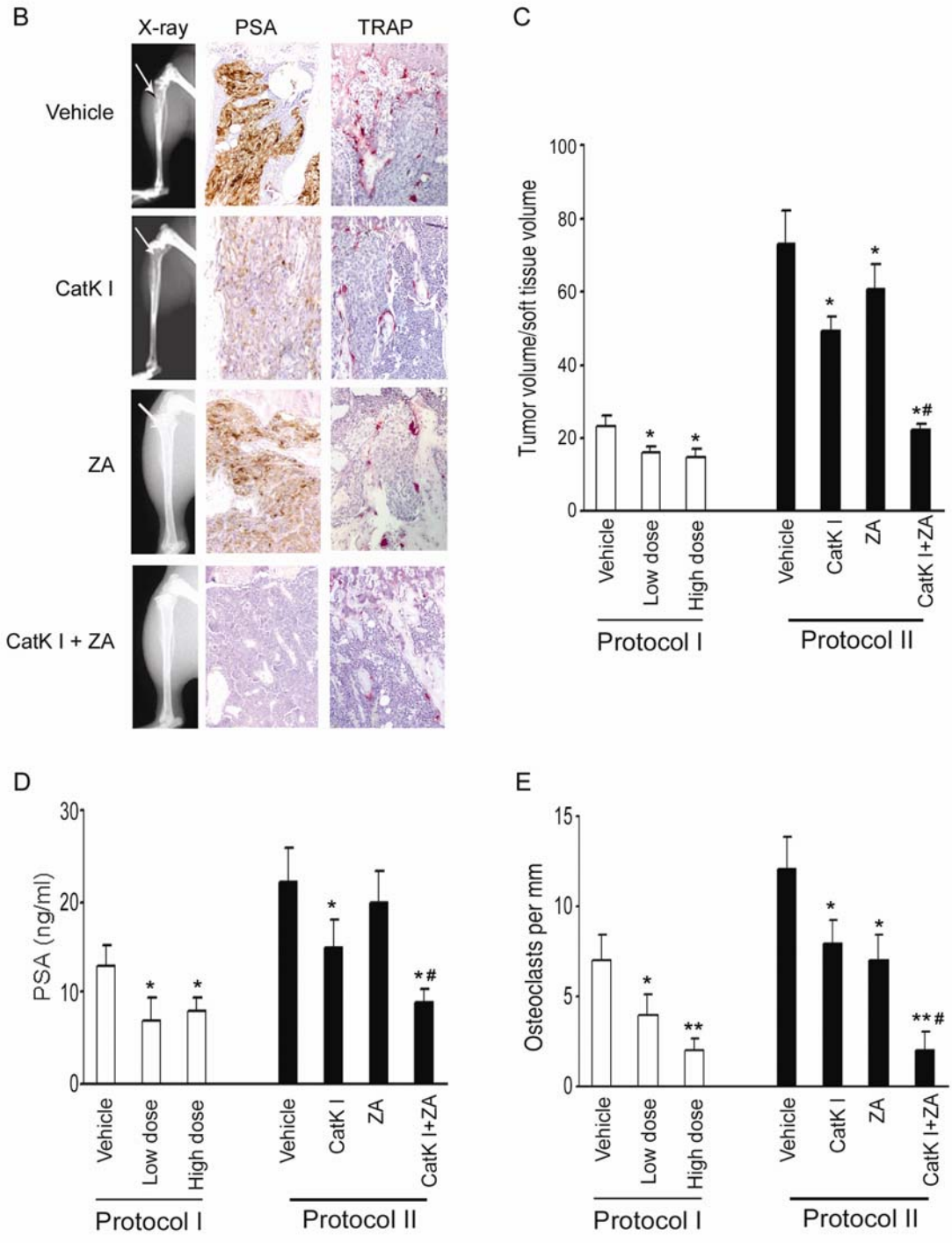
B
X-ray
PSA
TRAP
Vehicle
CatK I
ZA
CatK I + ZA
C
Tumor volume/soft tissue volume
100
80
60
40
20
0
Vehicle
Low dose
High dose
Vehicle
CatK I
ZA
CatK I+ZA
Protocol I
Protocol II
D
PSA (ng/ml)
30
20
10
0
Vehicle
Low dose
High dose
Vehicle
CatK I
ZA
CatK I+ZA
Protocol I
Protocol II
E
Osteoclasts per mm
15
10
5
0
Vehicle
Low dose
High dose
Vehicle
CatK I
ZA
CatK I+ZA
Protocol I
Protocol II

Figure 4B-E

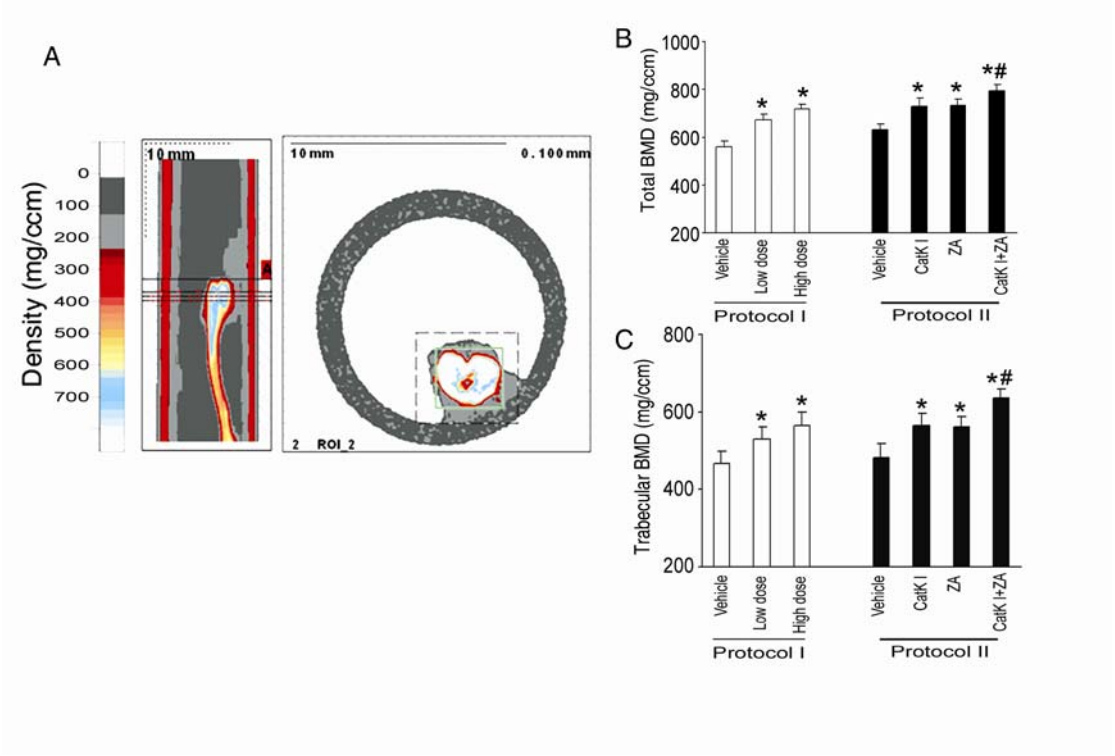
A
Density (mg/ccm)
0
100
200
300
400
500
600
700
10 mm
10 mm
0.100 mm
2 ROI_2
B
Total BMD (mg/ccm)
1000
800
600
400
200
Vehicle
Low dose
High dose
Vehicle
CatK I
ZA
CatK I+ZA
Protocol I
Protocol II
C
Trabecular BMD (mg/ccm)
800
600
400
200
Vehicle
Low dose
High dose
Vehicle
CatK I
ZA
CatK I+ZA
Protocol I
Protocol II

Figure 5

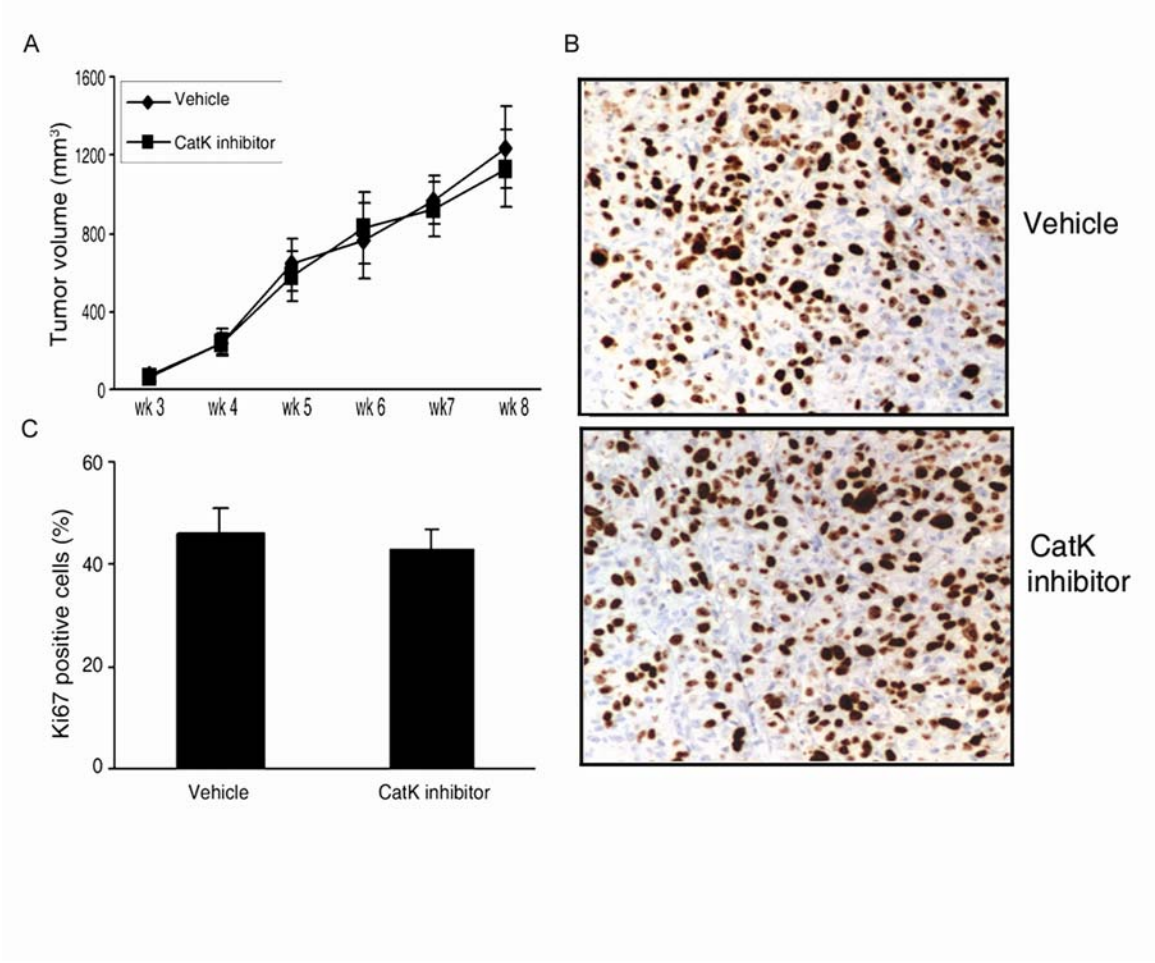
A
1600
1200
800
400
0
Tumor volume (mm3)
Vehicle
CatK inhibitor
wk 3
wk 4
wk 5
wk 6
wk7
wk 8
B
Vehicle
CatK
inhibitor
C
60
40
20
0
Ki67 positive cells (%)
Vehicle
CatK inhibitor

Figure 6